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Citation	Japanese Journal of Veterinary Research, 45(4), 217-220
Issue Date	1998-02-27
DOI	https://doi.org/10.14943/jjvr.45.4.217
Doc URL	https://hdl.handle.net/2115/2614
Type	departmental bulletin paper
File Information	KJ00002398580.pdf



Functional enucleation of mouse metaphase II oocytes with etoposide

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(Accepted for publication, Jan. 8, 1998)

Abstract

Mouse metaphase II (M II) oocytes were exposed to 50 $\mu\text{g/ml}$ etoposide (ETO) before and after parthenogenetic activation with 7% ethanol and they were washed with 0.75 M sucrose. The ETO treated parthenogenetically activated oocytes were cultured or fused to single blastomeres of late 2-cell stage mouse embryo to test their ability to support development *in vitro*. In parallel untreated parthenogenetically activated oocytes were cultured to serve as control. None of ETO treated oocytes developed beyond the 2-cell stage, whereas 4% of the reconstituted embryos and 35% of control developed to blastocysts. It is concluded that mouse M II oocytes can be functionally enucleated by ETO treatment and can be used for nuclear transfer experiments.

Key words : Enucleation, etoposide, reconstituted embryos

Currently etoposide (ETO), a specific DNA topoisomerase II inhibitor, is employed for chemical enucleation of murine oocytes^{4,6)}. Chemical enucleation will allow to overcome the problems of micromanipulation and to produce large numbers of recipient oocytes for nuclear transfer and nucleocytoplasmic interaction studies. Mouse metaphase I (M I) oocytes enucleated with ETO in the presence of cycloheximide, a protein synthesis inhibitor, were not able to support the development of reconstituted embryos beyond the 4-cell stage⁴⁾. DNA topoisomerase II is a structural component of metaphase chromosomes and is required for chromosomes separation, condensation and decondensation during mitosis^{1,2,15)}. DNA topoisomerase II is also known to cut the DNA strands⁹⁾. DNA topoisomerase II targeted drugs prevent the enzyme from releasing the cuts

in the DNA strands, and inhibit cell division¹⁵⁾. Blocking DNA topoisomerase II with teniposide induced DNA breaks during spermatogenesis in *Xenopus laevis*, and resulted in damage of the DNA and degeneration of spermatogonia B⁷⁾. Treatment of Surf Clam (*Spisula solidissima*) oocytes with the DNA topoisomerase II inhibitor (teniposide) for 1 hr prior to fertilization induced DNA breaks and inhibited development beyond the 2-cell stage¹⁵⁾. We anticipated that mouse M II oocytes can be functionally enucleated by blocking DNA topoisomerase II with ETO. The present study, tested the efficacy of exposure of mouse M II oocytes to ETO for the functional enucleation.

Oocytes were collected from six-week-old F1 female mice (C57B \times CBA) superovulated as described before⁴⁾. Superovulated mice were sacrificed by cervical dislocation 16 to 17 hr after

the hCG injection. The oviducts were excised and placed in a simplex optimization medium with an increased potassium concentration (KSOM)⁵⁾ covered with paraffin oil. The oviducts were transferred into Dulbecco's phosphate buffered saline (PBS) supplemented with 4 mg/ml bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, MO, USA) and containing 150 IU/ml hyaluronidase (Bovine testis type IS, H-3506, Sigma Chemical Co.). The ampullae were torn with a 25-gauge needle. The oocytes cumulus complexes were released, and left for 5 min in the PBS with hyaluronidase to remove the cumulus cells. The cumulus-free M II oocytes were washed in drops of KSOM and pooled until use.

Six-week-old ICR female mice were superovulated with PMSG and hCG and mated with males of the same strain. Late 2-cell stage embryos were collected by flushing the oviducts with KSOM at 46 to 48 hr after hCG injection. Zonae of the 2-cell stage embryos were removed with an acid Tyrode's solution (pH 2.7)⁸⁾. Blastomeres of 2-cell stage embryos were isolated by pipetting the zona-free embryos in Ca²⁺-free PBS supplemented with 6 mg/ml BSA.

To confirm that mouse M II oocytes can be functionally enucleated with ETO, they were cultured in KSOM containing 50 µg/ml ETO (Sigma chemical Co.) (KSOM+ETO) for 1 hr; thereafter, they were activated with 7% ethanol

in KSOM for 5 min¹⁴⁾, washed and cultured in 20 µl of KSOM containing ETO for 4 hr in multiwell plates (Sumitomo Bakelite Co., Tokyo, Japan) at 37°C in an atmosphere of 5% CO₂ in air. After this treatment, they were washed in KSOM and were exposed to 0.75 M sucrose at room temperature for 5 min. Some of the ETO treated parthenogenones were used to reconstitute embryos after removing their zona pellucidae with the acid Tyrode's solution and their polar bodies were also removed by pipetting. They were then aggregated with the isolated blastomeres in PBS containing 10 µg/ml phytohemagglutinin-P (Difco Laboratories, Detroit, MI, USA)¹¹⁾. The aggregated pairs were pipetted with a narrow-bore pipette to augment the area of contact between them. They were cultured in KSOM for 30 to 45 min and electrofused in PBS³⁾. The reconstituted embryos, ETO treated parthenogenones and control (parthenogenones not treated with ETO) were cultured in 20 µl of KSOM at 37°C in an atmosphere of 5% CO₂ in air. The cleavage rate was assessed 24 hr after the start of culture and the progress of development towards the blastocyst stage was recorded every 24 hr under an inverted microscope.

As shown in Table 1, very low proportion (5.3%) of ETO treated parthenogenones cleaved compared with the control (90.2%) and none of them developed to blastocysts. High proportion (89.4%) of the aggregated pairs fused and the

Table 1. Development *in vitro* of etoposide (ETO) treated parthenogenetic oocyte, reconstituted embryos and control

Treatment groups ^a	Total no. of oocytes		% of oocytes developed to ^b			
	treated	% fused	2-cell	4-cell	Morula	Blastocyst
ETO-Parthenogenone	141	—	5.3 ± 1.8 ^c	0.0 ^c	0.0 ^c	0.0 ^c
Reconstituted embryos	103	89.4 ± 2.3	87.6 ± 4.3 ^d	55.8 ± 5.5 ^d	40.2 ± 3.7 ^d	4.0 ± 2.3 ^c
Control-Parthenogenone	115	—	90.2 ± 1.7 ^d	87.8 ± 2.6 ^e	80.7 ± 6.2 ^e	35.5 ± 2.3 ^d

^a ETO-Parthenogenone: ETO treated parthenogenones washed with 0.75 M sucrose. Reconstituted embryos: ETO treated parthenogenones were washed with 0.75 M sucrose, aggregated with late 2-cell stage blastomeres and electrofused. Control-Parthenogenone: activated oocytes not treated with ETO.

^b Based on the total number of treated oocytes. Values were summarized as mean ± SEM of 4 replicates.

^{c-e} Values with different superscripts in the same column differ significantly (p < 0.0001).

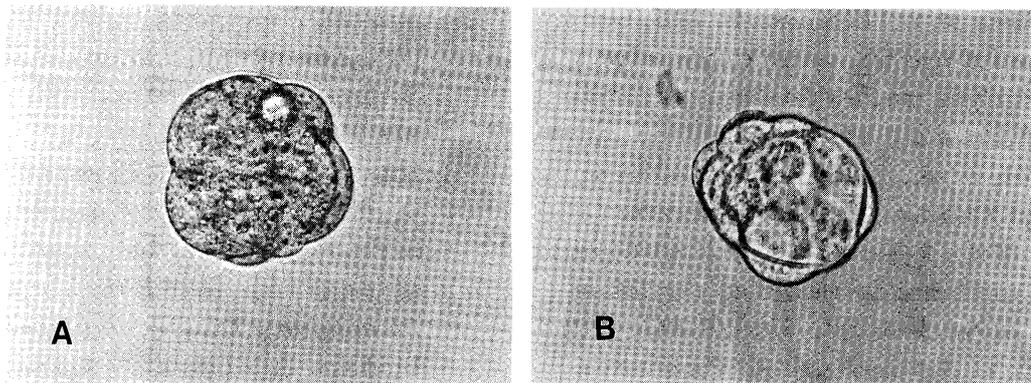


Fig. 1. A) Morula developed from reconstituted embryo obtained by fusion of late 2-cell stage blastomere to etoposide (ETO) treated parthenogenone. B) Early blastocyst developed from reconstituted embryo obtained by fusion of late 2-cell stage blastomere to ETO treated parthenogenone.

reconstituted embryos cleaved at a rate similar to that of the control (87.6%). The reconstituted embryos were able to develop to the 4-cell, morula and blastocyst stages (Fig. 1). However, their development rates were significantly lower ($p < 0.0001$, ANOVA followed by Fisher's PLSD-test) compared to those of the control.

Our results confirm that blocking DNA topoisomerase II before and after activation of mouse M II oocytes inhibits development beyond the 2-cell stage. This result suggests that the damage induced in the DNA by blocking DNA topoisomerase II denatures the metaphase chromosomes and inhibits the parthenogenetic development of mouse oocytes.

Reconstituted embryos obtained by fusion of late 2-cell stage blastomeres to cytoplasts obtained by bisection of pronuclear stage embryos are reported to develop to blastocysts at a rate of 15–20.7%¹³⁾. However, in our study a low proportion of blastocysts (4.0%) was obtained when late 2-cell stage blastomere were fused to parthenogenetically activated oocytes treated with ETO before and after activation. The discrepancy between the two studies may be due to the contributions of the abnormal nucleocytoplasmic ratio in our study¹⁰⁾, since we fused the 2-cell blastomeres to intact oocytes.

We can not ignore the indirect effects of blocking DNA topoisomerase II on development of the reconstituted embryos which is known to induce marked changes in histone kinases¹²⁾.

In conclusion, ETO treatment completely inhibits the parthenogenetic development of mouse oocytes, and the treated oocytes are able to support the development of late 2-cell stage blastomeres to blastocysts. This suggests that mouse M II oocytes can be functionally enucleated by blocking DNA topoisomerase II with ETO. However the normality of the reconstituted embryos needs to be confirmed in future studies by transfer of the reconstituted embryos to recipient animals.

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